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(54) **Method and compositions comprising a vitamin D derivative for the local treatment of bone fractures.**

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(73) Proprietor: **YEDA RESEARCH AND DEVELOPMENT COMPANY, LTD., Weizmann Institute of Science Herzl Street at Yavne Road P.O. Box 95, Rehovot 76100(IL)**

(72) Inventor: **Lidor, Cobi, 1 Avtalyon Street, Herzeliya 46 400(IL)**
Inventor: **Edelstein, Samuel, 23 Miller Street, Rehovot 76284(IL)**
Inventor: **Dekel, Samuel, 23 Barkai Street, Ramat Gan 52 376(IL)**
Inventor: **Meyer, Michael S., 13 Lipa Street, Tel-Aviv 69 712(IL)**

(74) Representative: **Brown, John David et al, FORRESTER & BOEHMERT Widenmayerstrasse 4/I, D-8000 München 22(DE)**

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In accordance with the present invention it has first been flound that local administration of $24,25(OH)_2D_3$ in vivo by injecting to the proximal cartilage growth plate of the tibiae of vitamin D-deficient chicks, resulted in disappearance of the rachitic lesions. Similar administration of $1,25(OH)_2D_3$ failed to show any sign of healing. These findings are consistent with previously published data suggesting that $24,25(OH)_2D_3$ is the most potent metabolite for healing rickets and probably plays a direct role in endochondral bone formation and that, furthermore, it is probably important for differentiation and maturation of the growth plate cartilage which has to be replaced by bone.

In the light of the above findings, another set of experiments was designed in order to demonstrate the effect of locally administered $24,25(OH)_2D_3$ in facilitating fracture healing. Experimental fractures were made in the mid-shaft of the tibiae of vitamin D-deficient chicks and a composition comprising 0.1% by weight of $24,25(OH)_2D_3$ in bone-wax was implanted at the frac- ture site. A control group of chicks were treated in the same manner except that at the fracture site there was implanted bone-wax without the active vitamin D_3 metabolite. A number of birds of both groups were killed 9 and 12 days after the fracture, calcium level in the plasma was determined and the calluses formed at the fractured tibiae were examined histologically. Both groups of chicks were found to be hypocalcaemic, indicating that the vitamin D_3 metabolite implanted at the fracture site did not enter the blood stream. It could be observed already macroscopically that the calluses formed after 9 and 12 days in the $24,25(OH)_2D_3$-treated chicks were considerably larger as compared to the control group, and after 12 days the two edges of the bones at the site of the fracture in the treated group were significantly less mobile than in the control fractures.

Histological examination of the calluses revealed three major differences between the control and the treated chicks:

(a) The cells in the calluses formed at the fracture sites of the control group were still undifferentiated cartilage cells of proliferative nature. In calluses formed in the $24,25(OH)_2D_3$-treated chicks, the majority of cells were well differentiated and hypertrophic.

(b) Relatively larger numbers of blood vessels were found in the calluses formed in the $24,25(OH)_2D_3$-treated group as compared with the control group calluses, and the appearance of mesenchymal cells (osteoblasts) and formation of osteoid were noted around vessels in the $24,25(OH)_2D_3$-treated calluses, while none were observed in the control group.

(c) In the $24,25(OH)_2D_3$-treated group, but not in the control group, there was observed formation of new trabeculae with mineralization.

A similar set of experiments was carried out on normal, vitamin D-supplemented chicks. The experimental protocol was identical to the one used with the vitamin D-deficient birds and similar results were observed.

The process of fracture-healing which is in principle a process of endochondral bone formation, involves the participation of various types of cells. The initial process in bone formation is the proliferation of the cartilage cells (chondroblasts). A distinct solid, extracellular matrix is being formed and the cells become hypertrophic. Mineralization appears in the extracellular matrix and thereafter the mineralized cartilage is resorbed by chondroclasts and mesenchymal and osteoblast cells appear and deposit new bone.

$24,25(OH)_2D_3$ was shown to affect the maturation, differentiation and function of cartilage cells (Corvol, M.T. et al., Endocrinology 102, 1269 (1978); Endo, J.H. et al., Nature 286, 262 (1980); Ornoy, A. et al., Nature 276, 517 (1978)). In the absence of this metabolite the maturation of cartilage cells is blocked, resulting in lack of endochondral ossification. One of the most important steps in the process of fracture healing is the formation of a cartilaginous callus in which proliferative cells are maturing to hypertrophic cells which are then replaced by bone, similar to the mechanism in the epiphyseal growth-plate. $24,25(OH)_2D_3$ locally applied to the site of the callus formation is most likely taken up by the cells at a very early stage in the process of callus formation, and the entire process is facilitated, as was indeed shown by the above experiments. Faster formation of the callus with faster maturation of the cells will, therefore, result in a faster healing of the fracture and repair of osteotomy.

$24,25(OH)_2D_3$ was shown in accordance with the present invention to affect the maturation and differentiation of cartilage cells, namely, the initial stages in the process of bone formation. However, several other factors that are known to affect cells participating in the later stages of said process could be advantageously used in order to further shorten the time required for fracture healing. Thus, local application of $1\alpha(OH)D_3$to and/or $1,25(OH)_2D_3$ in combination with $24,25(OH)_2D_3$ at the fracture site could result, not only in faster maturation of cartilage cells, but also in more rapid formation of bone due to an additional effect of $1,25(OH)_2D_3$ on osteoblast activity of these cells. Additional substances which may facilitate fracture healing if applied locally together with $24,25(OH)_2D_3$ at the fracture site include estradiol, hydroxyapatite crystals, fluoroapatite crystals and growth-hormone. All these factors are known to stimulate and facilitate fracture healing and bone formation.

Thus, the invention also contemplates within its scope a method for the treatment and promotion of healing of bone fractures and osteotomies in warm-blooded animals including humans, which comprise local applica tion to the site of the fracture or osteotomy of $24,25(OH)_2D_3$ in a suitable carrier in combina-

(a) 10 µl of arachis oil;
(B) 10 µl of arachis oil containing 3 µg of $24,25(OH)_2D_3$;
(c) 10 µl of arachis oil containing 1 µg of $1,25(OH)_2D_3$; and
(d) 10 µl of arachis oil containing 5 µg of 25-hydroxycholecalciferol ($25(OH)_2D_3$).

The oily solutions containing the vitamin D_3 metabolites were injected into the right tibia and the vehicle (arachis oil) only - into the left tibia.

Two aditional groups of chicks were studied and served as non-treated controls: a group of rachitic birds and a group of vitamin D_3-supplemented birds.

Following a course of three intraephiphyseal injections the chicks were killed and plasma was prepared for the determination of calcium and of vitamin D_3 metabolites. The proximal epiphyses of the tibiae were removed, fixed in 10% neutral formaldehyde, then dehydrated, and embedded in glycol methacrylate. Non-decalcified, 3 µm thick longitudinal sections were stained with toluidine blue or von Kossa stain and examined by light microscopy. The results are summarized in the following Table I.

As shown in Table I, the vitamin D-deficient chicks that were injected intraepiphyseally with $1,25(OH)_2D_3$ or with $24,25(OH)_2D_3$ retained low plasma concentrations of calcium, similar to untreated vitamin D-deficient birds. None of the hydroxylated vitamine D_3 metabolites could be detected in the plasma of these chicks, indicating that the injected metabolite did not enter the blood stream. However, the chicks that were injected intraepiphyseally with $25(OH)D_3$ showed normal plasma concentration of calcium and detectable levels of the hydroxylated metabolites, although significantly lower than the levels measured in the vitamin D-supplemented group.

The upper tibial epiphyses in all birds were still cartilaginous consisting of reserve, proliferative and hypertrophic zones. In the controls (+D) the different zones were well defined. Vitamin D-deficient chicks revealed typical signs of rickets, i.e. elongated proliferative and hypertrophic zones, wide osteoid seams with little mineralization of cartilage. However, it was difficult to clearly differentiate between the proliferative and hypertrophic zones and the measurements are related to total epiphyseal length only rather than to individual zones (see Table I).

The results prove that injection of $1,25(OH)_2D_3$ did not reduce the severity of the rickets, whereas injection of $24,25(OH)_2D_3$ caused almost complete recovery of the right tibia, while the left tibia still showed typical rachitic changes except for a slight reduction in total epiphyseal length. Injection of $25(OH)D_3$ was followed by recovery from rickets in both right and left tibiae although the recovery was more pronounced in the right tibia, the injected one. Furthermore, treatment with all metabolites resulted in a significant increase in the number of osteoclasts as compared to +D chicks. This was most prominent following $1,25(OH)_2D_3$ treatment.

EXAMPLE 6

Promotion of bone formation by local application of $24,25(OH)_2D_3$ in bone-wax to the site of the fracture

One-day old male chicks (Gallus domesticus) were depleted of vitamin D by feeding them a vitamin D-deficient diet for four weeks. Experimental fractures were performed under general anasthesia with continuous inhalation of Halotane. A skin incision was made under sterile conditions, the tibia was exposed and a transversal hole was drilled with the aid of a dental burr. Light pressure with the fingers caused transversal fracture of the bone. About 50 mg of bone-wax containing 5 µg of $24,25(OH)_2D_3$, prepared as described in Example 1 herein, was implanted at the fracture site on both edges of the fractures tibia. After 9 and 12 days some of the chicks were killed, plasma was prepared for calcium determination and the calluses of the fractured tibiae were removed, fixed in 10% neutral formaldehyde and prepared routinely for light microscopy. Decalcified preparations were made for Hematoxilyn and Eosin (H&E) staining and undecalcified preparations were made for von Kossa stain. A control group of chicks were treated in the same manner except that 50 mg of bone-wax which did not contain any $24,25(OH)_2D_3$ was implanted at the fracture site.

Both groups of chicks were hypocalcemic, indicating that the vitamin D_3 metabolite implanted at the fracture site did not enter the blood stream. The calluses formed after 9 and 12 days revealed macroscopically the formation of a larger callus at the fracture sites where $24,25(OH)_2D_3$ in bone-wax was implanted as compared to the control group. On the 12th day after the fracture it was further observed in the treated group that the two edges of the bones at the fracture site were significantly less mobile than in the control group.

The histological findings are illustrated in Figs. 1 to 18 of the accompanying drawings in which the following can be seen:

Figs. 1, 2 and 3: are microscopical views at magnifications of x70 (Figs. 1 and 2) and x280 (Fig. 3) of preparations taken from chicks of the D-deficient control group, 9 days after the fracture. In Figs. 1 and 3 (H&E stain) the appearance of the cartilage cells is the same, with very few blood vessels and no osteoblasts surrounding them. In Fig. 2 (von Kossa stain) there is no calcification in the cartilage area (C).

In Figs. 4 and 5 (magnification x70) and 6 and 7 (magnification x280) which were taken from the $24,25(OH)_2D_3$ -treated chicks 9 days after the fracture, there can be seen a large number of blood vessels (B in Figs. 4 and 5) within the cartilage milieu with the beginning of osteoid formation (+) and calcification ("Cal" in Fig. 7, von Kossa stain). The chondrocytes are of different sizes and shapes (Fig. 6).

In Figs. 8 to 10 which were taken from the D-deficient control group chicks 12 days after the fracture, there are still very few blood vessels (B in Fig. 9, magnification x280) with no differentiation in the cartilaginous tissue and no calcifications (Fig. 10, von Kossa stain, magnification x70).

Figures 11 to 18 were taken from the $24,25(OH)_2D_3$ -treated chicks, 12 days after the fracture, at magnifications x70 (Figs. 11, 12 and 15 to 17) and x280 (Figs. 13, 14 and 18). In Figs. 11 to 14 there can be observed chondrocytes in maturation (hypertrophic in Fig. 13) and many blood vessels in the cartilaginous milieu with osteoid surrounding them (in the centre of Fig. 14). In Figs. 15 (H&E stain) and 16 (von Kossa stain) new bone formation is seen and in Figs. 17 and 18 (von Kossa stain) - endochondrial ossification.

Table 2: Mechanical properties of the callus*

Group	n	Maximal Torque (N.m)$\times 10^{-2}$	Angle (degrees)	Initial Stiffness (N.m/deg.$\times 10^{3}$)	Stiffness (N.m/deg.$\times 10$
Normal∫	7	28.71±1.81**	33.3±2.6	10.25±0.82	8.84±0.71
Control 4	4	15.88±1.68	56.0±6.9	3.65±0.40	3.01±0.56
$1,25(OH)_2D_3$	6	13.89±1.83	62.5±5.3	3.24±0.38	2.24±0.24
$24,25(OH)_2D_3$	5	20.53±2.05	81.4±5.5	4.10±0.37	2.53±0.27
$1,25(OH)_2D_3$+$24,25(OH)_2D_3$	6	15.27±1.15	79.8±10.4	2.67±0.36	2.10±0.34

* Determined after 7 days of healing
** MEAN–SEM
∫ NORMAL – unbroken tibia

EXAMPLE 8

The effect of intra epiphyseal injection and local implantation of $24,25(OH)_2D_3$ on alkaline phosphatase activity

Introduction: Alkaline phosphatase activity serves as a biochemical parameter for bone formation (1), but its exact role is still unclear (2). In order to quantitate the changes in endochondral ossification which occur in the epiphyseal growth plate and in the callus after local administration of $24,25(OH)_2D_3$, we measured the alkaline phosphatase activity (AP).
Design of the experiments:

1. See Example 5 herein for the intraepiphyseal model of injection of $24,25(OH)_2D_3$.
2. See Example 6 herein for experimental callus formed in normal and -D chicks.

After 10 days (3 injections with 3 days intervals) in the intraepiphyseal injection model; and 7 days in the callus forming model, the chicks were killed. The epiphyseal growth plates and the calluses were dissected, weighed and homogenized with the aid of a Polytron homogenizer in ice-cold 0.15M NaCl containing 3 mM of $NaHCO_3$ (pH 7.4), and centrifuged at 20,000 x g for 15 min. at 4°C. The supernatants were assayed for alkaline phosphotase in 0.1M Sodium Barbital buffer, pH 9.3 with p-nitrophenyl phosphate (purchased from "Sigma") as a substrate. Protein was determined in the enzyme extracts by the Lowry method, and the results were expressed as units per mg protein.
One unit of phosphatase is defined as the enzyme activity that liberated 1 µmol of p-nitrophenol per 0.5h at 37°C.
Results and Discussion:

As shown in the following Tables 3 and 4 there is a marked reduction in the AP activity after local administration of $24,25(OH)_2D_3$.
The decreased activity of this enzyme correlates well with our findings (Table 5 below) when we determined AP activity in the epiphyses and diaphyses of normal chicks in comparison with rachitic birds; as well as with the published data in references (3,4). Shedden showed a loss of alkaline phosphatase activity close to a fracture site. This diminution of activity was not caused by the death of the cells in this region, close to the fracture, because a similar result was found in the periosteal cells in subperiosteal fractures (in which the periosteum was intact).
Conclusion: Local treatment with $24,25(OH)_2D_3$, either by direct injection to the epiphyseal growth plate or by implantation into the fracture site reduces alkaline phosphatase activity which is indicative of a better healing process.

(24,25-dihydroxycholecalciferol) in combination with a physiologically compatible vehicle suitable for use in orthopedic surgery for local application to a site of bone fracture or osteotomy, or for application to solid or semi-solid implants conventional in orthopedic surgery and to protheses, characterised in that the vehicle comprises bone wax, a bone cement or one of the components thereof, a bone sealant, demineralized bone powder, dacron mesh, gel-foam, or kiel bone.

2. A composition according to Claim 1 which comprises from 0.005 to 0.05% by weight of $24,25(OH)_2D_3$.

3. A composition according to Claim 1 or 2 wherein said carrier is a bone-wax.

4. A composition according to Claim 1 or 2 wherein said carrier is a bone cement or one of the components thereof.

5. A composition according to Claim 1 or 2 wherein said carrier is a bone sealant.

6. A composition according to Claim 1 or 2 wherein said carrier is a demineralized bone powder.

7. A composition according to Claim 1 or 2 wherein the carrier is a conventional orthopedic implant.

8. A composition according to Claim 7 wherein said implant is gel-foam.

9. A composition according to Claim 7 wherein said implant is dacron mesh.

10. A composition according to Claim 7 wherein said implant is kiel bone.

11. A composition according to any one of Claims 1 to 10 further comprising one or more auxiliary substances selected from $1a(OH)D_3$, $1,25(OH)_2D_3$, estradiol, hydroxy-apatite crystals, fluoro-apatite crystals and growth hormone.

12. Use of $24,25(OH)_2D_3$ (24,25-dihydroxycholecalciferol) for the manufacture of a medicament for the treatment and promotion of healing of bone fractures and/or osteotomies in warm-blooded animals by local application to a site of bone fracture or osteotomy.

Patentansprüche

1. Zusammensetzung für die Behandlung und die Unterstützung des Heilungsverlaufes von Knochenbrüchen und Osteotomien bei warmblütigen Tieren, einschließlich Menschen, die von 0,002 bis 0,2 Gew.-% $24,25(OH)_2D_3$ (24,25-Dihydroxycholecalciferol) in Kombination mit einem physiologisch verträglichen Träger umfaßt, geeignet zur Verwendung in der orthopädischen Chirurgie zur lokalen Anwendung auf einen Ort eines Knochenbruches oder einer Osteotomie oder zur Anwendung bei festen oder halbfesten Implantaten, die in der orthopädischen Chirurgie üblich sind, und bei Prothesen, dadurch gekennzeichnet, daß der Träger Knochenwachs, einen Knochenzement oder einen von dessen Bestandteilen, eine Knochendichtmasse, demineralisiertes Knochenpulver, Dacronnetz, Gelschaum oder Kielknochen ist.

2. Zusammensetzung nach Anspruch 1, dadurch gekennzeichnet, daß sie von 0,005 bis 0,05 Gew.-% $24,25(OH)_2D_3$ umfaßt.

3. Zusammensetzung nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß besagter Träger ein Knochenwachs ist.

4. Zusammensetzung nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß besagter Träger ein Knochenzement oder einer von dessen Bestandteilen ist.

5. Zusammensetzung nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß besagter Träger eine Knochendichtmasse ist.

6. Zusammensetzung nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß besagter Träger ein demineralisiertes Knochenpulver ist.

7. Zusammensetzung nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß der Träger ein übliches orthopädisches Implantat ist.

8. Zusammensetzung nach Anspruch 7, dadurch gekennzeichnet, daß besagtes Implantat Gelschaum ist.

9. Zusammensetzung nach Anspruch 7, dadurch gekennzeichnet, daß besagtes Implantat Dacronnetz ist.

10. Zusammensetzung nach Anspruch 7, dadurch gekennzeichnet, daß besagtes Implantat Kielknochen ist.

11. Zusammensetzung nach einem der Ansprüche 1 bis 10, weiter gekennzeichnet durch eine oder mehrere Hilfssubstanzen, ausgewählt aus $1a(OH)D_3$, $1,25(OH)_2D_3$, Östradiol, Hydroxylapatitkristallen, Fluorapatitkristallen und Wachstumshormon.

12. Verwendung von $24,25(OH)_2D_3$ (24,25-Dihydroxycholecalciferol) für die Herstellung eines Arzneimittels für die Behandlung und die Unterstützung des Heilungsverlaufes von Knochenbrüchen und/oder Osteotomien bei warmblütigen Tieren durch lokale Anwendung auf eine Stelle eines Knochenbruches oder einer Osteotomie.

Revendications

1. Composition pour le traitement et la stimulation de la cicatrisation des fractures osseuses et des ostéotomies chez les animaux à sang chaud y compris l'homme, qui comprend de 0,002 à 0,2% en poids de $24,25(OH)_2D_3$ (24,25-dihydroxycholécalciférol) en association avec un véhicule physiologiquement compatible, convenant pour l'utilisation en chirurgie orthopédique pour l'application locale sur l'emplace-

Fig. 2

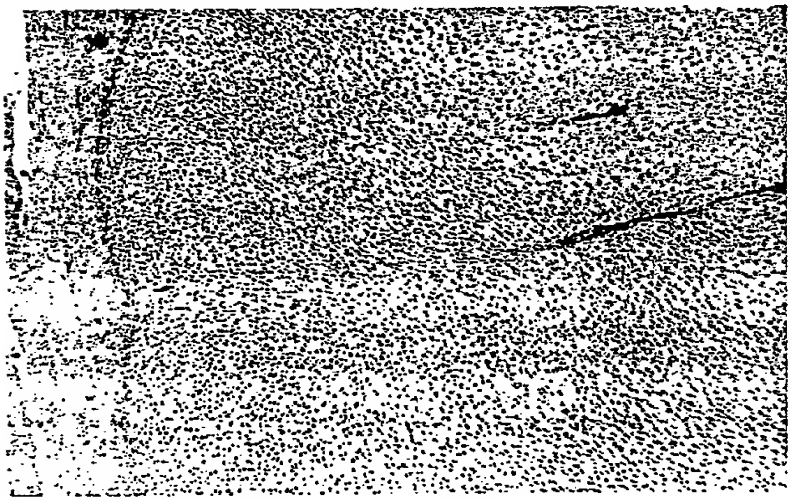

Fig. 1

Fig. 5

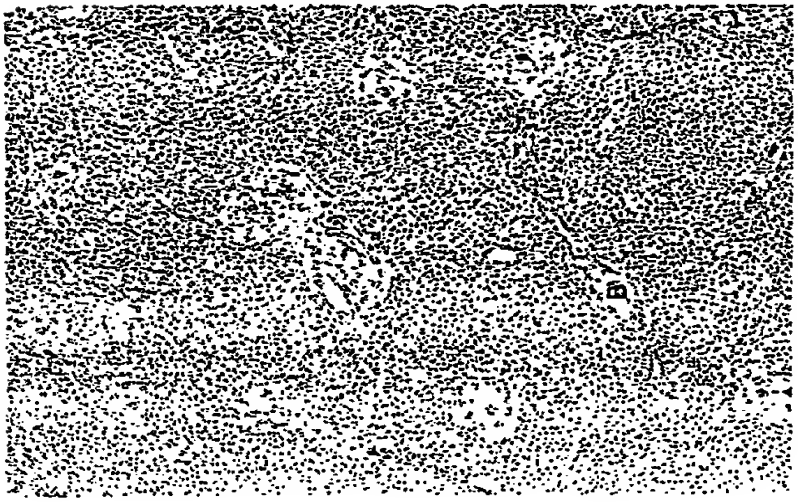

Fig. 4

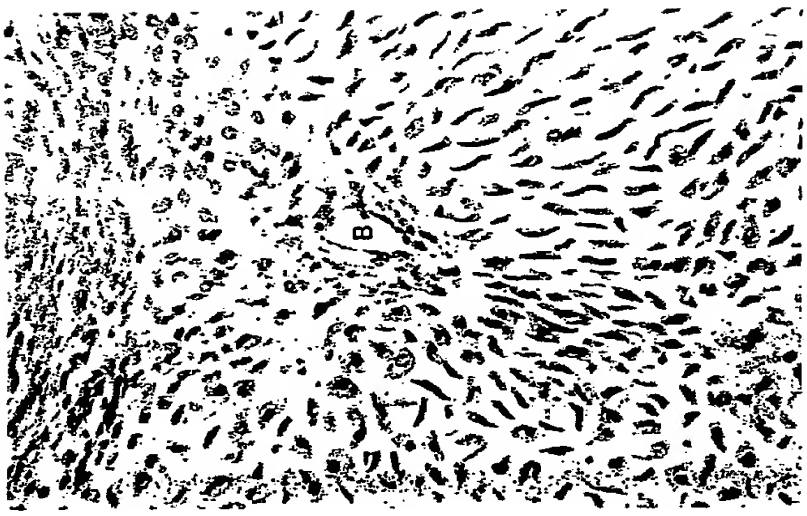

Fig.9

Fig.8

Fig.12

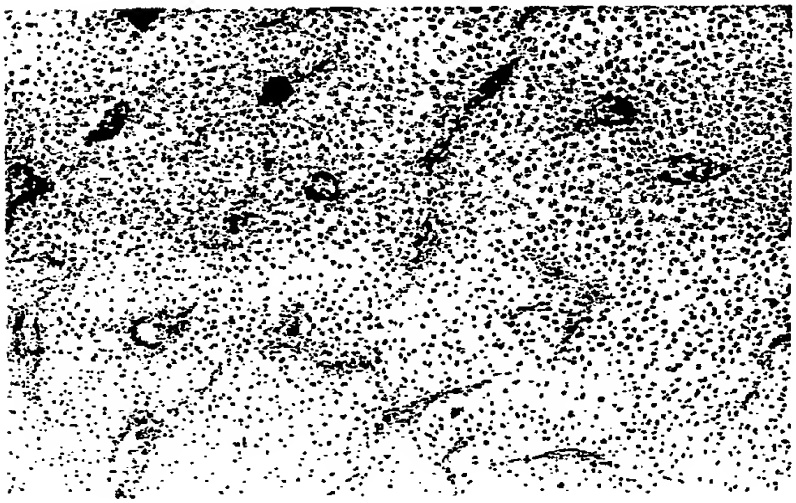

Fig.11

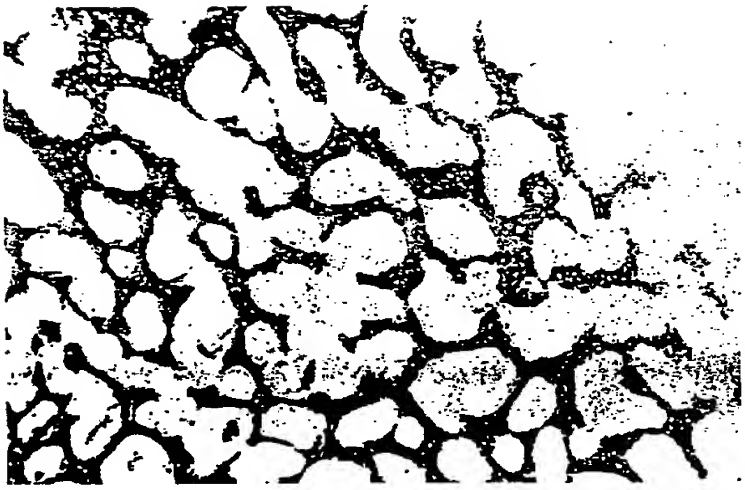

Fig. 16

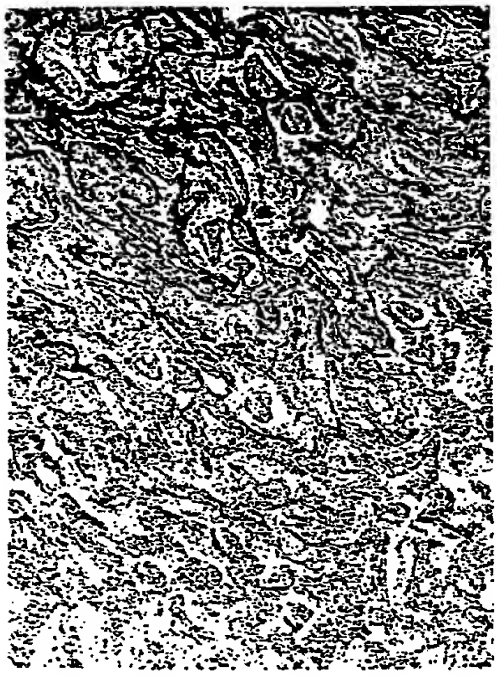

Fig. 15

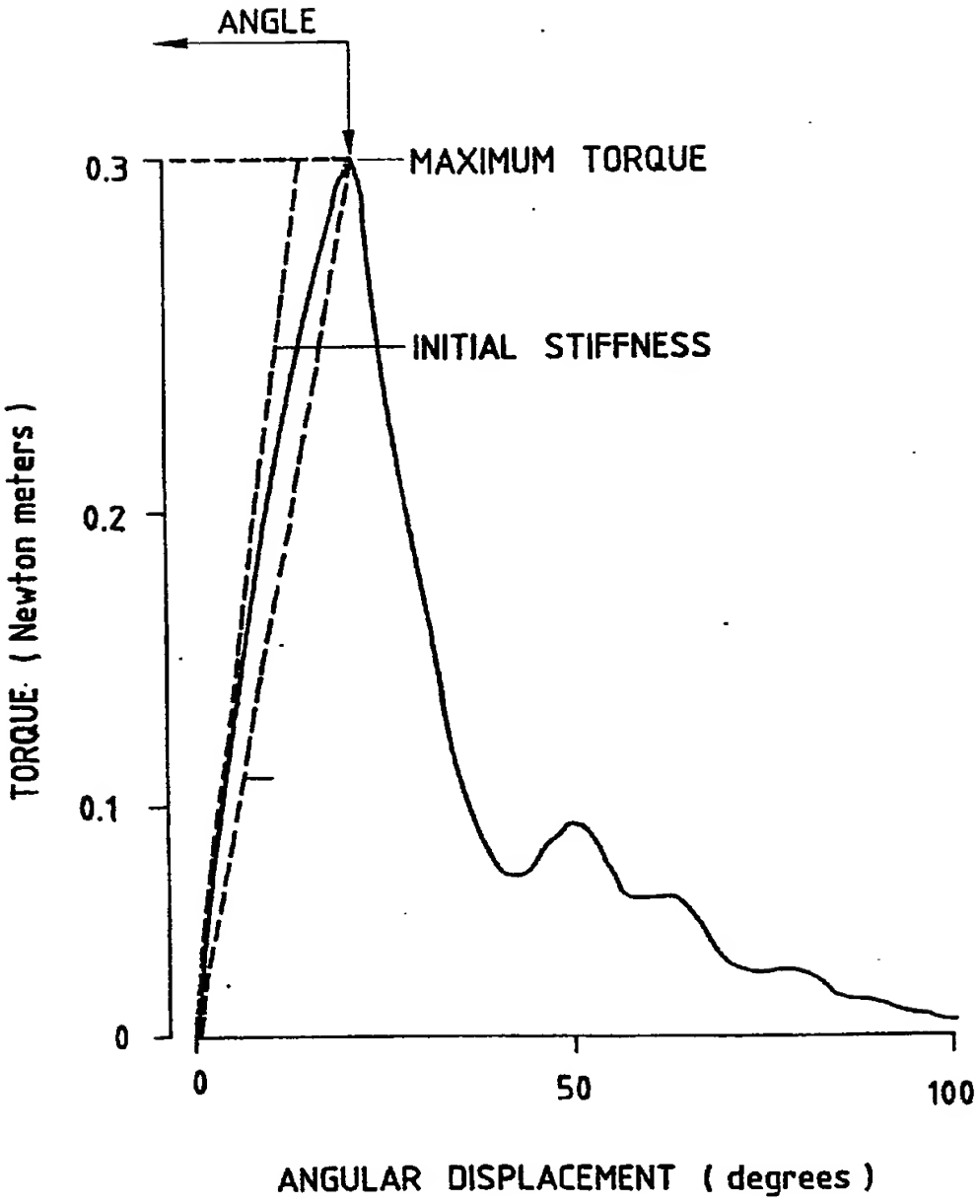
ANGLE
MAXIMUM TORQUE
INITIAL STIFFNESS
0.3
0.2
0.1
0
TORQUE (Newton meters)
0
50
100
ANGULAR DISPLACEMENT (degrees)

Fig. 19